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ORIGINAL ARTICLE

A mutation of the *Col2a1* gene (G1170S) alters the transgenic murine phenotype and cartilage matrix homeostasis

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KEYWORDS
cartilage;
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Background/Purpose: Genomic studies have revealed that there is a significant association between a point mutation of the human *Col2A1* gene (G1170S) and several hip disorders. The purpose of the study was to explore the phenotype and altered cartilage matrix homeostasis of transgenic mice carrying this mutated *Col2a1* gene.

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phenotype;
transgenic mice

Methods: Wild-type and transgenic mice were used as the control and study groups, respectively. Body weight measurement, radiographic analysis, and histological analysis of the mice were carried out to describe differences between the wild-type and transgenic mice at different ages. Cartilage metabolism studies were also carried out, including an MTT assay of cellular proliferation and nitric oxide and glycosaminoglycan assays. Allelic expression levels of the mutant A allele and the normal G allele were established by TaqMan assay. Cytokine and protease gene expression were measured.
Results: Transgenic mice had a lower mean body weight, a deformed skeletal structure, and abnormal cartilage histomorphology. Chondrocyte proliferation was significantly compromised and this was linked to significantly higher NO secretion and less soluble glycosaminoglycan formation. TNF-α and IL-1β gene expression was significantly upregulated, while MMP-13 gene expression was significantly downregulated.
Conclusion: The mutant G1170S *Col2a1* gene in mice clearly alters the transgenic murine phenotype and cartilage matrix homeostasis.

Introduction

The *Col2a1* gene encodes type II collagen, and mutation of this gene may cause type II collagenopathy. Type II collagen is by far the most abundant collagen in articular cartilage.[1] Articular cartilage provides a nearly frictionless surface and acts as a load absorber during movement. Type II collagenopathy prompts degradation of articular cartilage, which consequently results in arthropathies. Type II collagen also comprises most of the organic part of the physis.[2,3] During endochondral osteogenesis, long bone growth arises from cartilage, which is replaced by bone in the hypertrophic zone of the physis.[4] Type II collagenopathy disturbs the process of bone growth and this in turn causes abnormal skeletal development.

In cartilage, chondrocytes are surrounded by extracellular matrix (ECM).[5] The formation of type II collagen and glycosaminoglycan (GAG) favors ECM biosynthesis. ECM maintenance requires a balance between its biosynthesis and degradation, and this is regulated by chondrocytes. A number of potential catabolic factors may interact with chondrocytes and induce signaling cascades that activate gene transcription and post-transcriptional modifications.[6] Proinflammatory cytokines, including tumor necrosis factors (TNFs) and interleukin (ILs), collagen-degrading matrix metalloproteinases (MMPs), aggrecanases (ADAMTSs), excessive mechanical stress, reactive oxygen species (ROS), and degraded ECM particles are all catabolic factors that promote ECM degradation. In this context, the modulating factors secreted from chondrocytes are regulated if the structural components of the ECM change either towards biosynthesis or towards breakdown.

Mutation in the human *Col2a1* gene has been associated with many different skeletal abnormality patterns. The clinical phenotypes range from perinatal lethality due to achondrogenesis II and hypochondrogenesis, to relatively mild symptoms due to late-onset arthropathy, such as arthropathy with epiphyseal dysplasia, osteochondrodysplasia, and precocious osteoarthropathy.[4,7] In animal models, several forms of *Col2a1* point mutation result in various abnormal murine phenotypes.[8,9] In these studies, ultrastructural analyses revealed reduced collagen density, derangement of type II collagen fibrils, and the presence of dilated Golgi bodies. In parallel, radiographic studies showed shortened limbs and cleft palates, while histological studies have demonstrated disorganized growth plates.

The G1170S point mutation of human *Col2a1* is related to various hip disorders, including osteonecrosis of the femoral head (ONFH), Legg—Calvé—Perthes disease, and precocious osteoarthritis of the hip.[10—13] A new type II collagenopathy has recently been identified by histological and ultrastructural studies of cartilage of the human femoral head harvested during total hip arthroplasty in patients with a mutated gene.[14] However, no animal model with this *Col2a1* mutation has been reported to date.

There is a high degree of similarity between murine and human genomic sequences and this is supported by the fact that the numbers of protein-coding genes (~25,000 in mouse and ~24,200 in human) are also similar; therefore, mice are regarded as an ideal animal model for studying human genetic disorders. The human and murine Col2a1 amino acid sequences are highly similar. BLAST sequence comparison reveals 95% identity between the proteins encode by human *COL2A1* (NCBI accession number NP_001835.3) and murine *Col2a1* (NP_112440.2). The glycine residue at position 1170 and its neighboring amino acid are the same in both species. We used wild-type (WT) and transgenic (TG) G1170S *Col2a1* mice from the National Health Research Institutes of Taiwan as experimental animals. The purpose of the study was to elucidate whether the mutated *Col2a1* gene alters the murine phenotype and cartilage matrix homeostasis.

Methods

Introduction of a point mutation in *Col2a1*

The animal handling protocol was approved by the institutional committee of the National Health Research Institutes, Taiwan. To obtain a fragment of the *Col2a1* gene that contained serine at amino acid position 1170, a murine *Col2a1* cDNA clone obtained from a pCMV-SPORT6.1 library of mammalian genes (MGC; clone list accession number

BC052326) was purchased from Open Biosystems (Waltham, MA, USA) and used for PCR mutagenesis. Sense and antisense mutagenesis primers designed from the sequence of exon 48 with mutated nucleotides (in boldface type) were used, namely 5′-ACC TGG CCC TGT **T**AG TCC CTC TGG CAA AG-3′ and 5′-CTT TGC CAG AGG GAC **T**AA CAG GGC CAG GT-3′. Using the methods described in the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), a G→A single-base point mutation at position 3425 of the murine *Col2a1* cDNA sequence, which changes GGT (glycine) to AGT (serine), was introduced into the MGC clone. After verification of the mutation by sequencing, the PCR product was digested with EcoRI and Xbal, and the 4.8-kb mutant *Col2a1* cDNA fragment was subcloned into a pBlueScriptII KS(−) vector (Stratagene), and ligated to an SV40 polyA signal and two tandem units of the 1.2-kb insulator of the chicken globin gene.[15] The resulting construct was ligated to the 6.02-kb NotI—XboI fragment of a Col2a1-Cre gene construct (pCol2.IRES.cre.pA)[16] consisting of a 3-kb sequence of the *Col2a1* promoter region (exon 1 with a mutated initiation codon) and a 3.02-kb fragment of the first intron ligated to a splice acceptor sequence. The final 16.6-kb plasmid construct (pCol2A1-Ins-pA-G1170S) was digested with BssHII and the resulting 13.8-kb fragment was purified. This was microinjected into pronuclei of murine embryos from the inbred C57BL/6 mice strain.

Identification of transgenic mice

Transgenic mice were identified by PCR analysis of genomic DNA from the tail. The primers used for PCR were sense primer 3F-2 (5′-CTG CAC CAT CTC TTC CAG-3′) for exon 40 and antisense primer 4R (5′-CAA AGG TGT TCG AGG AGA C-3′) for exon 43 (Fig. 1A). The predicted PCR product size is 371 bp for the transgene and 950 bp for the endogenous *Col2a1* template. Therefore, the products were easily distinguished after separation by agarose gel electrophoresis (Fig. 1B). The transgene copy number was determined by Southern blotting. In brief, extracted DNA was digested with EcoRI, separated by agarose gel electrophoresis, transferred to a nylon membrane, and probed with a [^{32}P]dCTP-labeled 1.2-kb insulator sequence complementary to the end part of the transgene. The expected DNA fragment size is 4.4 b. Heterozygous transgenic progeny for this study were obtained from crosses between two WT females and the male founder. The WT and TG strains in both cases were C57BL/6 mice. All mice were bred in a specific pathogen-free facility.

Morphological measurements and radiographic imaging

Eight WT mice and four TG mice were used for morphological and radiographic studies. The body weight of each mouse was recorded from 13 to 31 days after birth. At the age of 12 weeks, the mice were sacrificed and radiographs were obtained.

Histology of femoral head cartilage

Femoral heads were harvested from WT and TG mice at the ages of 2 months and 10 months. Hematoxylin and eosin staining was performed. The arrangement and morphology of chondrocytes and their zonal distribution in articular cartilage were compared.

Chondrocyte culture

Mice of 4—6 days of age were sacrificed by CO_2 asphyxiation. Cartilage pieces were taken from the knees of WT and TG mice and were minced and washed in sterile PBS twice, then treated with 0.2% collagenase and incubated in 5% CO_2 at 37°C. After complete digestion, the chondrocytes were collected and seeded in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) in 10-mm dishes and allowed to grow to confluence. Chondrocytes in passage 2 were used as cells for spectrophotometric assays. The phenotype of the cultured chondrocytes was verified by alcian blue straining, which specifically stains GAG.

MTT assay

For MTT assays, 6250 chondrocytes/well were introduced into 96-well microtiter plates for cell culture. At different time points (Days 1, 4, 7, and 14), the mitochondrial activity of cells was determined by colorimetric assay of the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) to insoluble formazan. At each time point, a sample of cell supernatant was removed 25 μL of 2.5 mg/mL MTT solution was added to each well. The wells were incubated at 37 °C for 3.5 hours to allow formazan crystals to form. The supernatant was removed and 100 mL of 0.04 N HCl in ethanol was added to each well and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on an ELISA plate reader (Spectra max 340, Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

NO assay (Griess reaction)

For NO assays, 6250 chondrocytes/well were introduced into 96-well microtiter plates for cell culture. At different time points (Days 1, 4, 7, and 14), 20 μL of medium from each well was transferred to another 96-well microtiter plate and 10 μl of 0.1% sulfanilamide solution and 10 μl of 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride were added to each well. After incubation for 10 minutes in the absence of light, a purple color developed. The absorbance of the converted dye was measured at 550 nm within 30 minutes using an ELISA plate reader.

GAG assay

For GAG assays, 6250 chondrocytes/well were introduced into 96-well microtiter plates for cell culture. At different time points (Days 1, 4, 7, and 14), 20 μL of medium from each well was transferred to another 96-well microtiter plate and 125 μL of dimethylmethylene blue solution was added to each well. The absorbance of the converted dye was measured at 525 nm using an ELISA plate reader.

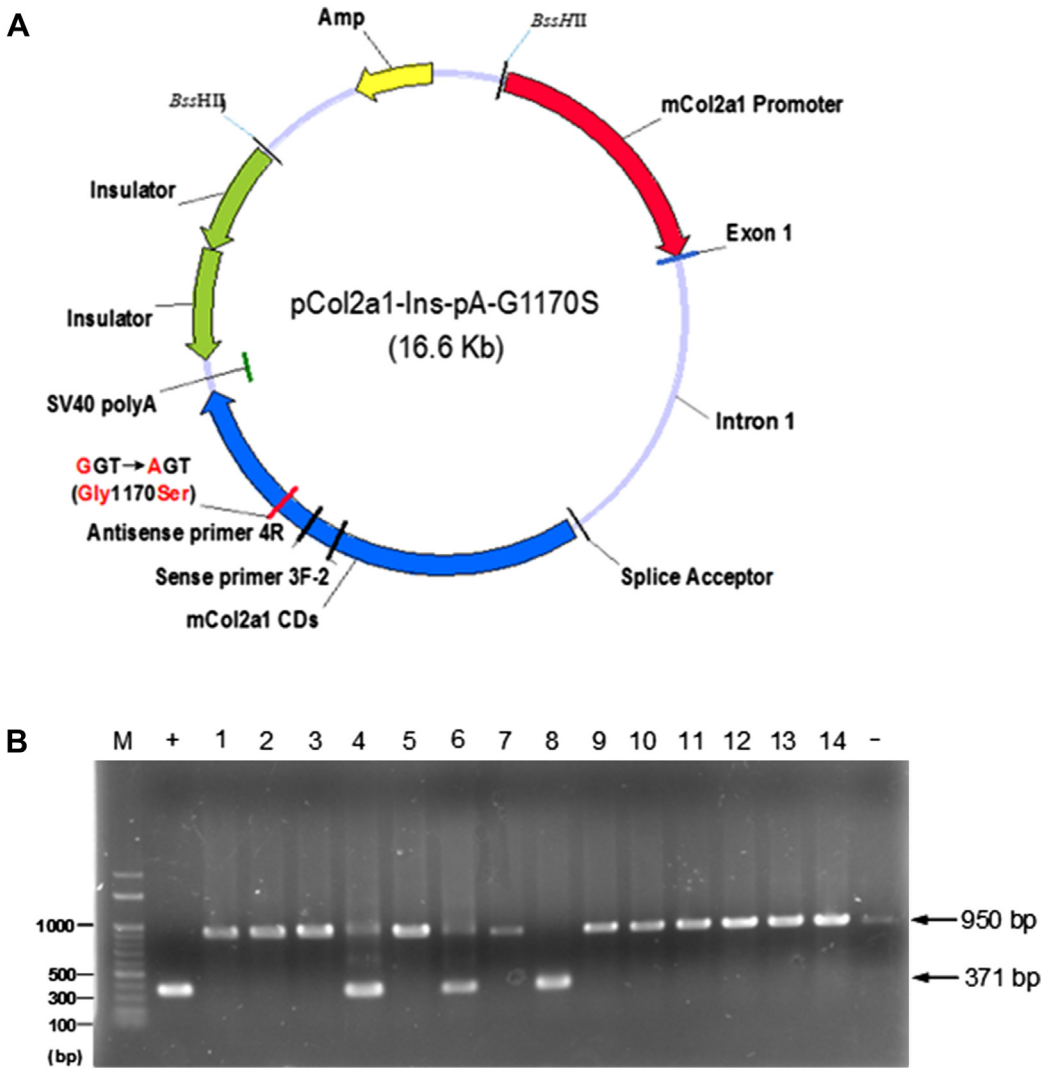

Figure 1 Transgene construct and genotyping. (A) Transgene construct. The 16.6-kb mutant construct consists of the promoter region of the *Col2a1* gene (mCol2a1 Promoter); a *Col2a1* first intron (Intron 1) that directs chondrocyte-specific expression of the transgene murine *Col2a1* coding sequence (mCol2a1 CDs); an SV40 polyA signal (SV40 polyA); and two tandem units of the insulator of the chicken β-globin gene (Insulator). The function of the insulator is to overcome any positional effects with respect to transgene expression. The BssHII sites were used to excise the 13.8-kb insert for pronucleus microinjection. Sense primer 3F-2 and antisense primer 4R targeting *Col2a1* exons 40 and 43 were used for genotyping. The position of the G → A point mutation in exon 48, which changes Gly to Ser at amino acid 1170, is shown by a red line. (B) Genotyping. The predicted size of the PCR products differs between the genomic template (950 bp) and the transgene template (371 bp). PCR genotyping was able to identify lanes 4, 6, and 8 as TG mice. The other lanes were recognized as WT mice. Lane+, transgene template. Lane−, genomic template.

TaqMan allelic discrimination

Pieces of cartilage were dissected from the knee joints of 4-day-old WT and TG mice and were snap-frozen in liquid nitrogen and homogenized. Total RNA was isolated using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. TaqMan assays were carried out using a two-step RT-PCR protocol and a high-capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies, Grand Island, New York, USA). The sequences for the primers and allele-specific probes ware as follows: TaqMan sense primer, 5′-TGG TCC TTC TGG CCC TAG AG-3′; TaqMan antisense primer, 5′-GGA TTC CAT TAG AGC CAT CTT TGC-3′; WT allele-specific G probe, FAM-5′-CCC TGT TGGT CCC TC-3′; and mutant allele-specific A probe, VIC-5′-CCC TGT TAG TCC CTC-3′. PCR amplification was carried out on an ABI 7000 system (Applied Biosystems) at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The β-actin gene was amplified as an endogenous control to standardize the sample loadings. Data analysis was performed using an ABI Prism 7000 instrument (Applied Biosystems) set to universal RT-PCR parameters. Allelic expression was compared for the normal G allele in WT cells (WT-G) and in transgenic cells (TG-G) and the mutant A allele in transgenic cells (TG-A).

Quantitative real-time PCR analysis

Quantitative real-time PCR analysis was used to examine the expression level of various proinflammatory factors (TNF-α, IL-1β, and iNOS) and of various matrix-degrading enzymes (MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5). Total RNA was isolated from dissected knee-joint cartilage pieces from 4-day-old WT and TG mice, and cDNA was synthesized as described in the previous section. The primer sequences are shown in Table 1. Thermocycling and data analysis were performed using an ABI Prism 7000 instrument. Four independent experiments were carried out for each sample for both the TaqMan allelic discrimination assay and quantitative real-time PCR analysis. Data obtained for the target gene were normalized against the housekeeping gene β-actin for each group. ΔCt and $\Delta\Delta$Ct values were calculated according to $\Delta Ct = \Delta Ct_{\text{target gene}} - \Delta Ct_{\beta\text{-actin}}$ and $\Delta\Delta Ct = \text{mean } \Delta Ct_{\text{study group}} - \text{mean } \Delta Ct_{\text{control group}}$. The expression ratio for target genes in the study group relative to that in the control group was quantified using the $2^{-\Delta\Delta Ct}$ approach.

Statistics

All data were analyzed using SPSS Statistics 17.0 (IBM, International Business Machines Corp., Armonk, New York, USA). Data are expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) followed by a least squares difference (LSD) *post hoc* test was applied for three-group comparison of TaqMan allelic discrimination data. The Student *t* test was used to compare data for the other assays. A *p* value of <0.05 was considered statistically significant.

Results

Morphological measurements and radiographic imaging

Mean body weight was lower for TG than for WT mice at all time pints, but none of the differences were significant (Fig. 2A). The extent of spinal kypholordosis was greater in TG than in WT mice. The vertebral body was shorter and broader in TG than in WT mice. Furthermore, the contour of the occipital skull was more sharply angled in TG than in WT mice (Fig. 2B,C). There was no obvious radiographic evidence of osteonecrosis or osteoarthritis in the heterozygous TG mice.

Cartilage histology

At the age of 2 months, there was a larger calcified zone present in the femoral head cartilage of TG mice compared to WT mice (Fig. 3A). In 10-month-old TG mice, the gross articular cartilage thickness was less than that in WT mice of the same age; furthermore, the chondrocyte density was lower in these TG mice compared to their WT counterparts. Microscopically, chondrocyte morphology in 10-month-old TG mice was different and more disoriented compared to that in WT mice of the same age (Fig. 3B).

MTT assay of chondrocytes

The presence of *Col2a1* G1170S in TG chondrocytes affected their viability as measured by MTT assay when compared to WT chondrocytes (Fig. 4A).

NO assay

The presence of *Col2a1* G1170S in TG chondrocytes affected NO synthesis as measured by NO assay. The amount of NO associated with chondrocytes was significantly higher for TG than for WT mice on Days 1 and 14 (Fig. 4B).

GAG assay

The presence of *Col2a1* G1170S in TG chondrocytes affected their soluble GAG production. The amount of

Table 1 Gene primers used for quantitative real-time PCR analysis.

Gene	Primers		Product size (bp)
TNF-α	Forward	5′-CCAGACCCTCACACTCAGA-3′	79
	Reverse	5′-CACTTGGTGGTTTGCTACGA-3′	
IL-1β	Forward	5′-CAACAAGTGATATTCTCCATG-3′	103
	Reverse	5′-CACAGGACAGGTATAGATTC-3′	
iNOS	Forward	5′-AACATCAGGTCGGCCATCA-3′	88
	Reverse	5′-CGTACCGGATGAGCTGTGA-3′	
MMP-3	Forward	5′-AATACTGGAGGTTTGATGAGA-3′	127
	Reverse	5′-AAGTAGAGAAACCCAAATGCT-3′	
MMP-9	Forward	5′-GTGTCTGGAGATTCGACTTG-3′	102
	Reverse	5′-AGATGTCGTGTGAGTTCCAG-3′	
MMP-13	Forward	5′-CTTGTGTTTGCAGAGCACTA-3′	89
	Reverse	5′-ACTGTGGAGGTCACTGTAGA-3′	
ADAMTS-4	Forward	5′-GACTTCAATGTTCCTCAGGC-3′	138
	Reverse	5′-ACAATACTTGCCACCGTTCC-3′	
ADAMTS-5	Forward	5′-TGAGCCAATAATGCCGTCAC-3′	100
	Reverse	5′-TCCAAAGGTTACGGATGGGA-3′	
β-Actin	Forward	5′-CCATCATGAAGTGTGACGTTGA-3′	104
	Reverse	5′-CTTCTGCATCCTGTCAGCAATG-3′	

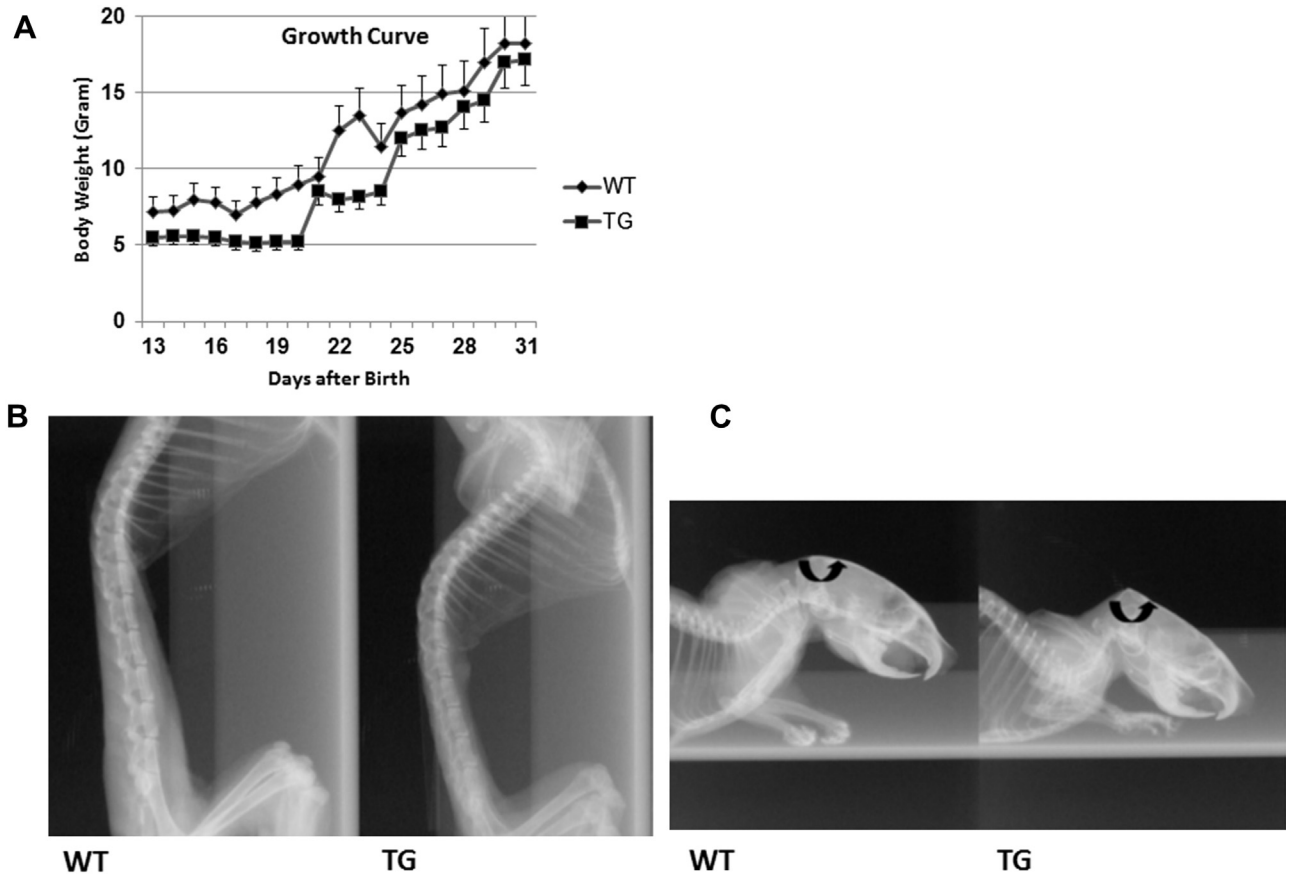

Figure 2 Growth curve of and skeletal changes in the transgenic mice. (A) Growth curve chart. Solid triangles and squares denote the mean body weight for WT and TG mice, respectively. Only positive error bars for WT mice and negative error bars for TG mice are shown to avoid overlap of negative WT and positive TG error bars at the same time point. Mean body weight was greater for the eight WT mice than for the four TG mice at all time points but the difference was not significant over the time period measured. (B) Radiographic lateral view of the thoracolumbar spine. TG mice had more significant kyphosis along the thoracolumbar spine and broader and shorter vertebral bodies than the WT mice. (C) Radiographic lateral view of the skull and upper spine. TG mice showed more significantly sharp-angled contouring of the occipital skull (right curved arrow) than WT mice (left curved arrow). More significant lordosis of the upper spine in TG than in WT mice is also evident.

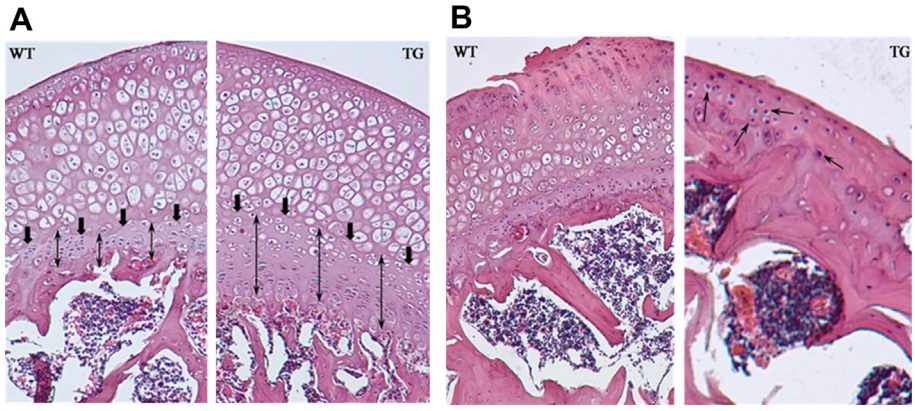

Figure 3 Histological studies of murine femoral heads. (A) Histological analysis of the femoral head at age 2 months (×100). The calcified zone (double-headed arrows) of the articular cartilage is located beneath the tidemark (bold arrows). The proportion of the calcified zone in the whole cartilage layer is larger for TG than for WT mice. (B) Histological analysis of femoral head cartilage at age 10 months (×100). The articular cartilage thickness is less in TG mice, and chondrocyte density and size (arrows) are also less in TG mice. The discrimination between different zones is more obscure in TG mice. The stratified and columnar patterns of chondrocyte arrangement in superficial and middle zones are disoriented, and irregular arrangement of chondrocytes is evident in TG mice.

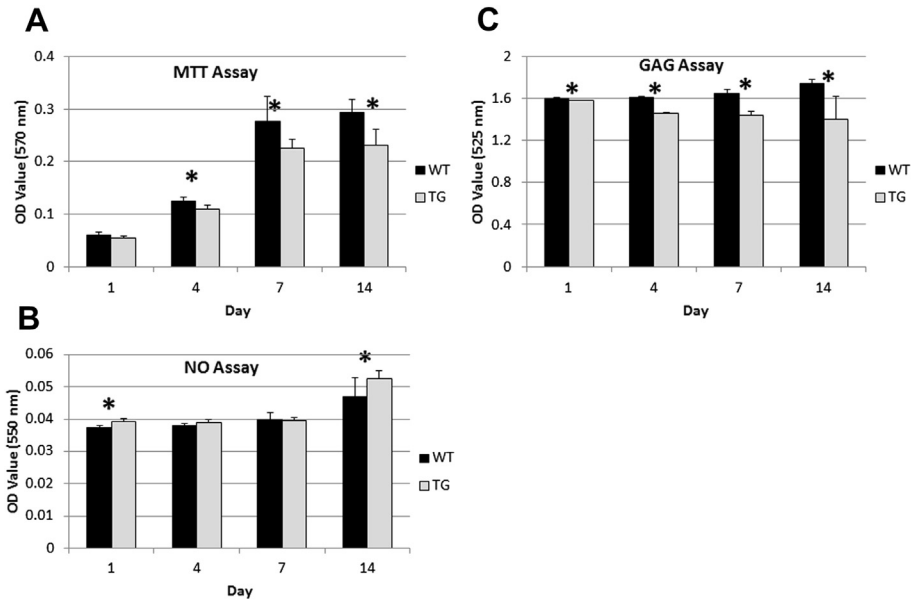

Figure 4 MTT, NO and GAG assays. (A) MTT assay of chondrocyte proliferation. Data are shown as mean ± SD. $N = 8$ for each group on Days 1 and 4; $N = 7$ for each group on Day 7; $N = 6$ for each group on Day 14. *A p value of <0.05 is considered significant. Chondrocyte proliferation was significantly lower in the TG group than in the WT group on Day 4 ($p = 0.01$), on Day 7 ($p = 0.03$), and on Day 14 ($p = 0.002$). (B) NO assay to quantify ROS. Data are shown as mean ± SD. $N = 8$ for each group on Days 1, 4, 7, and 14. * A p value of <0.05 is considered significant. The level of NO released by chondrocytes was significantly higher in TG than in WT mice on Day 1 ($p = 0.001$) and Day 14 ($p = 0.04$). (C) GAG assay to quantify GAG production. Data are shown as mean ± SD. $N = 8$ for each group on Days 1, 4, 7, and 14. * A p value of <0.05 is considered significant. The level of GAG produced by chondrocytes was significantly lower in TG than in WT mice for all four time points ($p = 0.033$, $p < 0.001$, $p = 0.001$, $p = 0.006$, respectively).

soluble GAG in culture medium was significantly lower for TG than for WT chondrocytes at all four time points (Fig. 4C).

Allele-specific *Col2a1* mRNA expression

The mean ΔCt value for WT-G, TG-A, and TG-G alleles was −1.62, −1.55, and −0.33, respectively. For WT-G versus TG-G and TG-A versus TG-G the difference in ΔCt is significant (both $p < 0.001$). However, the ΔCt difference between TG-A and WT-G is not significant ($p = 0.54$). The allelic expression ratio was 0.95 for TG-A relative to WT-G, 0.41 for TG-G relative to WT-G, and 2.33 for TG-A relative to TG-G (Fig. 5A).

Transcriptional profiles of proinflammatory factors, collagen-degrading enzymes, and aggrecanases

Mean TNF-α mRNA expression in TG mice was 1.46 times that in WT mice and the difference in ΔCt between the groups was significant ($p < 0.001$). Mean IL-1β mRNA expression in TG mice was 1.77 times that in WT mice and the difference in ΔCt between the groups was significant ($p = 0.012$). There was no significant difference in mRNA expression levels of iNOS ($p = 0.13$), MMP-3 ($p = 0.82$), and MMP-9 ($p = 0.72$) between TG and WT mice. Mean MMP-13 mRNA expression in TG mice was 0.85 times that in WT mice and the difference in ΔCt between the groups was significant ($p = 0.04$). There were no significant differences in ADAMTS-4 ($p = 0.40$) and ADAMTS-5 ($p = 0.39$) mRNA expression between WT and TG mice. These results indicate that mRNA expression of TNF-α and IL-1β was significantly upregulated, while mRNA expression of MMP-13 was significantly downregulated in TG compared to WT mice (Fig. 5B,C).

Discussion

Phenotypic studies of mice carrying various *Col2a1* mutations have revealed alterations in the cartilage matrix, reductions in the density and arrangement of type II collagen fibrils, the presence of dilated Golgi bodies, disorganization of growth plates, the presence of shortened limbs and cleft palates, and a proportional reduction in body size.[9,17–19] Radiographic analysis of TG mice carrying the *Col2a1* Arg519Cys mutant revealed primary osteoarthritis and osteochondrodysplasia, which is similar to the skeletal abnormalities found in humans with the same mutation.[20] In our study, we observed gross differences between WT and TG mice that affected their growth and led to visible changes on radiographic images. Mean body weight was lower for TG mice and radiographic analysis demonstrated an increased kypholordotic spinal curve and shorter and broader vertebral bodies. These changes are similar to radiographic characteristics associated with human osteochondrodysplasia, which is known to occur for several *Col2a1* mutations.

Histological analysis of samples from our TG mice revealed an increased calcified zone in younger TG mice, in accordance with a histological study of humans carrying

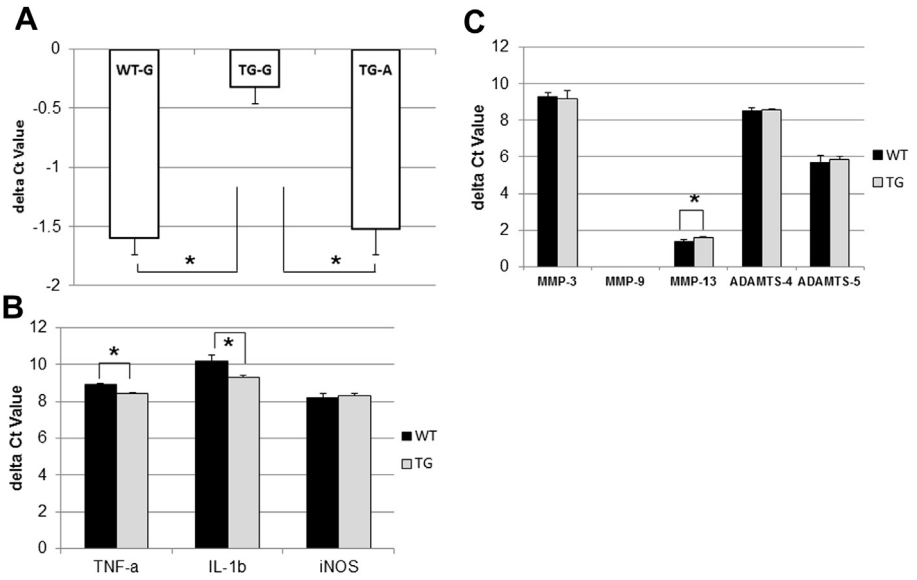

Figure 5 Gene expression levels. (A) Allele-specific *Col2a1* mRNA expression. WT-G, normal *Col2a1* G-allele expression in WT mice; TG-G, normal *Col2a1* G-allele expression in TG mice; TG-A, mutant *Col2a1* A-allele expression in TG mice. Data are shown as the ΔCt value (mean ± SD, $N = 4$). *A p value of <0.05 is considered significant. A lower ΔCt value indicates greater genetic expression. Significant ΔCt differences were found for TG-G versus WT-G and for TG-A versus TG-G (both $p < 0.001$). No significant ΔCt difference was detected between TG-A and WT-G ($p = 0.54$). The allelic expression ratio for the study group relative to the control group was calculated according to the $2^{-\Delta\Delta CT}$ method. Allelic expression ratios were 0.95 for TG-A relative to WT-G, 0.41 for TG-G relative to WT-G, and 2.33 for TG-A relative to TG-G. The mutant A allele apparently replaces the normal G allele and is dominant in TG mice. (B,C) Transcriptional profiles of various proinflammatory factors, various collagen-degrading enzymes, and various aggrecanases. Data are shown as the ΔCt value (mean ± SD, $N = 4$). * A p value of <0.05 is considered significant. A lower ΔCt value indicates greater genetic expression. The genetic expression ratio for the TG group relative to the WT group was calculated using the $2^{-\Delta\Delta Ct}$ method if a significant difference in ΔCt existed between the groups. mRNA expression levels of TNF-α and IL-1β were significantly upregulated in TG mice. The mRNA expression level of MMP-13 was significantly downregulated in TG mice. Genetic expression ratios for TNF-α, IL-1β, and MMP-13 in TG mice relative to those in WT mice were 1.46, 1.77, and 0.85, respectively.

the same *Col2a1* point mutation.[14] Microscopic alterations in femoral head cartilage might cause changes in bulk mechanic properties and even diminish the ability to tolerate stress, making femoral head cartilage more vulnerable to stress during daily activity. For older TG mice, histological analysis identified thinner articular cartilage, a lower chondrocyte density, changes in chondrocyte morphology, and irregularities in chondrocyte arrangement, all of which are associated with cellular degeneration. This might partly explain the presence of precocious osteoarthritic hips in humans carrying the *Col2a1* G1170S mutation.[14] We found that expression of the normal G allele was significantly downregulated in our heterozygous TG mice, and the mutant A allele seemed to have replaced the normal G allele as the dominant allele responsible for type II collagen production. In the transcription process, the *Col2a1* transgene lacking introns was transcribed more efficiently than the normal G allele in TG mice because of competition for the transcription machinery and cofactors in cells. This might be the reason why the normal G allele was downregulated in TG mice and replaced by the mutant A allele.

It has been shown that NO, a proinflammatory mediator, is related to the prevalence of osteoarthritis (OA) and rheumatoid arthritis (RA).[21] NO plays roles in various processes, including inhibition of ECM biosynthesis, enhancement of ECM degradation, promotion of cellular injury, and promotion of chondrocyte susceptibility to cytokine-induced apoptosis, all of which suggest that NO acts as a catabolic factor.[22] NO release was significantly higher in TG than in WT mice on both Day 1 and Day 14. Furthermore, soluble GAG production was significantly higher in WT than in TG mice at all time points. In effect, there is a roughly inverse relationship between mean NO secretion and mean soluble GAG production in TG and WT mice, and therefore we suggest that NO release is related to inhibition of GAG biosynthesis.

TNF-α and IL-1β are proinflammatory cytokines that mediate the differentiation of osteoclasts, inducing osteolysis in rheumatoid arthritis, osteolysis in periodontal disease, and aseptic loosening of prostheses.[23] They also induce synovial fibroblasts and articular chondrocytes to produce proteases that can degrade the cartilage matrix.[24] The mRNA expression of these proinflammatory cytokines was significantly upregulated in TG mice. This parallel upregulation of TNF-α and IL-1β gene expression in articular cartilage, together with increased secretion of NO, probably affects the neighboring subchondral bone. The immune response caused by diffusion of these proinflammatory cytokines and mediators might lead to osteolysis of the subchondral bone, which is relatively hypervascular compared to the covering cartilage. This proposed process agrees with what is known about the initial pathological lesions of ONFH and Legg—Calvé—Perthes disease. These are located in femoral

subchondral bone and are highly likely to be related to the human *COL2A1* G1170S mutation. In addition to these changes, the MTT assay revealed significantly compromised chondrocyte proliferation in TG mice. This might be a result of enhanced chondrocyte degeneration due to synergistic effects of significant TNF-α and IL-1β upregulation and increased production of NO. Such changes in chondrocyte degeneration might also be associated with the histological findings of degeneration-like phenomena in femoral head cartilage from older TG mice.

Interestingly, MMP-13 mRNA expression by chondrocytes from TG mice was significantly downregulated relative to WT mice. Inhibition of this collagen-degrading enzyme might represent the initiation of a healing process under the influence of various catabolic factors, such as upregulation of TNF-α and IL-1β expression and increased production of NO. However, chondrocyte mRNA expression levels of MMP-3, MMP-9, ADAMTS-4, and ADAMTS-5 were not significantly different between TG and WT mice. Articular cartilage is an avascular connective tissue and the local immune response induced by upregulation of TNF-α and IL-1β and increases in NO secretion might not be strong enough to induce significant ECM degradation. According to Southern blotting, the copy number for the mutant transgene was only one (data not shown) and the normal *Col2a1* gene was still present in TG mice. In such circumstances, the impact of the mutant gene might not be strong enough to cause significant alterations in the expression of MMP-3, MMP-9, ADAMTS-4, and ADAMTS-5 matrix-degrading enzymes in TG mice.

The absence of the WT 950-bp fragment in lane 8 of the genotyping analysis is interesting (Fig. 1). This missing fragment might result from competition during PCR, whereby more abundant templates compete with less abundant ones for reagents. In the PCR amplification step, the short 371-bp TG template was generated more efficiently than the long 950-bp WT one, resulting in consumption of most the reagents. Consequently, trace amounts of the 950-bp WT fragment might not be visible on the agarose gel.

In conclusion, the phenotype of TG mice with the mutant *Col2a1* G1170S gene includes skeletal and chondral abnormalities, as well as compromised proliferation of chondrocytes. Furthermore, the mutant A allele acts as a dominant marker and enhances the gene expression of various pro-inflammatory factors.

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